# Retinoid X Receptor-Specific Ligands Synergistically Upregulate 1, 25-Dihydroxyvitamin $D_3$-Dependent Transcription in Epidermal Keratinocytes *In Vitro* and *In Vivo*

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**We have examined the mechanism by which endogenous retinoid X receptor (RXR), vitamin $D_3$ receptor (VDR), and cognate ligands regulate nuclear 1,25-dihydroxyvitamin $D_3$ ($D_3$) signaling in epidermal keratinocytes from skin, a physiologic $D_3$ target. *In vitro*, RXR and VDR-specific antibodies identified endogenous RXR and VDR bound to a vitamin $D_3$-responsive element (DR3) as heterodimers (VDR-RXR). In cultured keratinocytes, 9-*cis* retinoic acid (9cRA), a panagonist for RXR and retinoic acid receptor (RAR), and an RXR-selective agonist, SR11237, synergized with $D_3$ to activate DR3 *via* endogenous as well as overexpressed VDR-RXR, whereas both of these RXR agonists alone were ineffective. In contrast, SR11237 did not synergize with but antagonized an RAR-selective ligand activation of a retinoic acid-responsive element (DR5) *via* endogenous RAR-RXR. Furthermore, expression of RXR mutated in transactivation domain AF-2 inhibited endogenous VDR-RXR activity over DR3. This mutant efficiently bound to DR3 as VDR-RXR but showed reduced capacity to transactivate DR3 in response to $D_3$ and SR11237. *In vivo*, $D_3$ and SR11237 synergistically induced the naturally occurring $D_3$-responsive 24-hydroxylase gene in epidermis of mouse skin, whereas SR11237 alone was ineffective. Our data suggest that allosteric changes caused by VDR in DR3-bound VDR-RXR do not block access of ligands to RXR. RXR ligand-induced conformational changes permit VDR-RXR, *via* both VDR and RXR activation function domains, to mediate maximal $D_3$ signaling in keratinocytes. *Key words: 9-cis retinoic acid/24-hydroxylase/vitamin $D_3$ receptor. J Invest Dermatol 108:506–512, 1997***

The retinoid X receptor (RXR) gene family consists of three members, $\alpha$, $\beta$, and $\gamma$, and is one of two nuclear receptor families mediating the biologic effects of retinoids, the other being the retinoic acid receptor (RAR) family. RARs and RXRs are structurally related, but have distinct ligand specificity (reviewed in Chambon, 1996). 9-*cis*-Retinoic acid (9cRA) is a natural ligand of RXRs and also recognizes RARs. In the presence of 9cRA, RXRs can form homodimers and activate transcription from gene promoters containing RXR-responsive elements (RXRE). RXRs are also known to form heterodimers with other members of the steroid/thyroid hormone receptor superfamily such as RAR, vitamin $D_3$ receptor (VDR), thyroid hormone receptor, and peroxisome proliferator-activated receptor. The resulting dimers regulate gene transcription through binding to corresponding *cis*-acting hormone-responsive elements, termed RARE, VDRE, TRE, and PPARE, respectively, located in the transcription regulatory region of target genes.

Skin is a target tissue for retinoids and vitamin $D_3$. Both hormones differently regulate epidermal growth and differentiation, which are accompanied by changes in expression of several growth and differentiation-related genes (Bikle and Pillai, 1993, and references therein). RXR, RAR, and VDR are believed to mediate the retinoid and vitamin $D_3$ effects, respectively (Bikle and Pillai, 1993; Ross *et al*, 1994, and references therein). A recent study has shown that RXRE is not activated by RXR ligand in epidermal keratinocytes due to the lack of RXR homodimers and competitive formation of RAR-RXR heterodimers (Xiao *et al*, 1995). Moreover, the ligand responsiveness of RAR-RXR heterodimer-mediated transactivation has been found to be regulated by RAR-selective ligands but not RXR-selective ligands (Xiao *et al*, 1995). It is not known, however, whether in KCs the RXR ligand, 9cRA, regulates the ligand responsiveness of VDR-RXR heterodimers bound to VDRE of type DR3, which is composed of two directly repeated consensus half-sites spaced by 3 bp. It has been previously observed that the 9cRA responsiveness of the DR3-bound VDR-RXR heterodimers varies among various cell lines, suggesting the existence of cell type-specific regulation of vitamin $D_3$ signaling by 9cRA (Carlberg *et al*, 1993; MacDonald *et al*, 1993; Schrader *et al*,

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Abbreviations: 9cRA, 9-*cis* retinoic acid; AF-2, transactivation function-2; $D_3$, 1,25-dihydroxyvitamin $D_3$; DR3, directly repeated consensus hexameric half-sites separated by 3 bp; DR5, directly repeated consensus hexameric half-sites separated by 5 bp; RAR(s), retinoic acid receptor(s); RXR(s), retinoid X receptor(s); dnRXR, dominant negative RXR mutant; RARE(s), retinoic acid responsive element(s); VDR, vitamin $D_3$ receptor; VDRE(s), vitamin $D_3$-responsive element(s).

1993; Cheskis and Freedman, 1994; Ferrara *et al*, 1994; Schrader *et al*, 1994; Sasaki *et al*, 1995).

In the present study, we examined not only interaction among VDRE(DR3) and endogenous VDR and RXR in epidermal keratinocytes from human skin but also the roles of RXR and its ligand 9cRA in 1,25-dihydroxyvitamin $D_3$ ($D_3$)-dependent transactivation. To exclude possible promiscuous effects caused by binding of 9cRA to RARs and interconversion in keratinocytes between 9cRA and its natural stereoisomer, RAR-selective all-*trans*-RA, RXR-selective synthetic retinoid SR11237 (Lehmann *et al*, 1992) was also used in this study. We observed that endogenous VDR and RXRs in keratinocyte nuclear extracts prepared directly from adult human epidermis bound *in vitro* to VDRE(DR3) as heterodimers. Reporter gene assays showed that both 9cRA and SR11237 upregulated $D_3$-dependent transactivation of this element by VDR-RXR heterodimers in keratinocytes cultured in serum-free, defined media. Furthermore, the ligand-dependent activation function-2 (AF-2) of RXR in VDR-RXR was found to be required for the RXR ligand effect. In good correlation with our *in vitro* observations, topical application of SR11237 and $D_3$ to mouse skin synergistically induced expression of a naturally occurring $D_3$-target gene, 1,25-dihydroxyvitamin $D_3$ 24-hydroxylase, in epidermis whereas SR11237 alone had no effect. Our data clearly indicate that in epidermis, RXR-specific retinoids upregulate the $D_3$ signaling pathway *via* VDR-RXR heterodimers and that RXR within VDR-RXR is not only a DNA-binding co-factor but also a transcriptional activator.

## MATERIALS AND METHODS

**Ligands** 1,25-Dihydroxyvitamin $D_3$ was kindly provided by Dr. M. Uskokovic, and 9-*cis*-retinoic acid, by Drs. P. F. Sorter, J. F. Grippo, and A. A. Levin (Hoffmann-La Roche, Nutley, NJ). CD367 was generously provided by Dr. B. Shroot (CIRD, Sophia Antipolis, Valbonne, France) (Martin *et al*, 1992). SR11237 (81104-BASF), which was originally synthesized by Dr. M. I. Dawson (Lehmann *et al*, 1992), was provided by Dr. B. Janssen (BASF-Aktiengesellschaft, Ludwigshafen, Germany).

**Reporter Gene Plasmids and Expression Vectors for VDR, RXR, and a Dominant Negative RXR Mutant** The reporter gene VDRE-tk-chloramphenicol acetyl transferase (CAT) was constructed by inserting double-stranded oligodeoxyribonucleotides, 5′-tcgact **AGGTCA** AGG **AGGTCA** gaga-3′ (consensus hexameric half-sites shown in *bold capital letters* and random nucleotides in *lowercase letters*) containing a consensus VDRE(DR3) (Umesono *et al*, 1991) into polycloning sites located upstream of the minimal promoter from the HSV thymidine kinase gene in pBS-tk-CAT (Xiao *et al*, 1995). Reporter gene, $\beta RARE_3$-tk-CAT, contains three head-to-tail copies of βRARE(DR5) from the hRARβ2 gene located immediately upstream of tk-CAT in pBLCAT8+ (Xiao *et al*, 1995). Expression vectors for the wild-type and dominant negative RXRα, pSG5-mRXRα, and pSG5-mRXRαdn were described previously (Xiao *et al*, 1995). The dominant negative RXRα mutant contains a limited deletion at its C terminus (amino acid positions 449–467) and is referred to as dnRXR hereafter. pXJ40-hVDR, an expression vector for VDR, was constructed by inserting the human VDR cDNA fragment from p91023B (Baker *et al*, 1988) into the polycloning sites of pXJ40, a cytomegalovirus enhancer/promoter-driven expression vector (Xiao *et al*, 1995).

**Human Skin Epidermal Biopsies and Keratinocyte Culture** As described by Xiao *et al* (1995), epidermal keratome biopsies were obtained from buttock skin of healthy adult human volunteers. As keratinocytes represent approximately 95% of cells constituting epidermis (Voorhees *et al*, 1972), the results from human epidermis described in this study predominantly pertain to receptors present in epidermal keratinocytes. Primary cultures of KCs from these individuals were derived, and subcultures were expanded in a defined serum-free medium, KGM, purchased from Clonetics (San Diego, CA). All procedures involving handling human subjects have been approved by the University of Michigan Institutional Review Board, and each volunteer provided written consent.

**Nuclear Extract Preparation and Gel Mobility Shift Assays** Nuclear extracts from epidermal keratinocytes of keratome biopsies and cultured KCs were prepared as described by Xiao *et al* (1995). Regular and immunologic gel mobility shifts were carried out as previously described (Rochette-Egly *et al*, 1991) with modification by Xiao *et al* (1995). Oligonucleotide probes containing the wild type (VDREwt) or mutant (VDREmut) VDRE(DR3) were labeled at the 5′-end using γ-[$^{32}$P]-ATP (6000 Ci/mmol, DuPont-NEN, Boston, MA) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Sequences of these probes are 5′-tcgact **AGGTCA** AGG **AGGTCA** gaga-3′ for VDREwt and 5′-tcgact **AAGTCA** AGG **AAGTCA** gaga-3′ (mutations *underlined*) for VDREmut. A mouse monoclonal antibody against RXRs used in immunologic gel mobility shifts was 4RX, which specifically recognizes an epitope in the DE region of all three RXR subtypes (α, β, and γ). For analyzing VDR, a rat monoclonal antibody, MA1–710 (Affinity BioReagents, Neshanic Station, NJ), was used. This antibody was raised against the DNA-binding domain of VDR.

**Overexpression of VDR, RXRα, and dnRXR in Cultured Keratinocytes** Cultured keratinocytes at the stage of third passage (about 80% confluent) were transfected by lipofection with a total 12.5 μg per ϕ100-mm dish of expression vector DNA for VDR, RXRα, or dnRXR (at 1:1 ratio in the case with co-overexpression of two different receptors). Transfected keratinocytes were fed with fresh KGM 18 h after transfection and harvested 48 h thereafter. Nuclear extracts were prepared as described above.

**Transfection of Keratinocytes and CAT Assays** Keratinocytes at the stage of the second passage were passed and seeded into ϕ60-mm dishes. When cell density reached about 80% confluence, 2 μg of a reporter gene plasmid alone or together with 100 ng of each receptor expression vector were introduced by lipofection into the cells. Lipofect'ACE was used as a lipofection reagent according to the instruction provided by the manufacturer (Life Technologies, Bethesda, MD). Eighteen hours after the lipofection, cells were treated with fresh KGM containing vehicle (0.1% ethanol) or appropriate ligands for 48 h before harvesting. Prior to CAT assays, variations in transfection efficiencies were normalized based on β-galactosidase activity generated from a co-transfected constitutive expression vector containing the bacterial *lacZ* gene, pXJ40-LacZ (2 μg), as described (Xiao *et al*, 1995). Each experiment was repeated with KCs from a different individual who represents a sample number (n) of one.

**Mouse Strain and Topical Treatment of Mouse Skin with Ligands** Normal C57BL/6 adult mice were purchased from Jackson Laboratory (Bar Harbor, ME). Skin hairs were removed using a Golden A-5 clipper (Oster Professional Products, McMinnville, TN) prior to topical treatments. $D_3$ and/or SR11237-containing solutions were prepared using acetone as a vehicle. Eight hundred microliters of the solution were applied topically to the entire mouse skin once per day for 3 d before animals were sacrificed for preparation of epidermal keratome biopsies. All procedures in which mice were used received approval from the University of Michigan Committee on Use and Care of Animals.

**Preparation of Mouse Epidermal RNA and Northern Blotting Analysis** Mouse epidermal biopsies were taken using a keratome device with a blade set at 0.2 mm to cut near the junction of epidermis-dermis. Thus, biopsies contained primarily epidermis with residual amount of dermis (approximately 10% maximum). Biopsies were snap-frozen immediately in liquid nitrogen. Total RNA was then isolated from the biopsies as described by Elder *et al* (1991). Twenty micrograms of RNA from each treated mouse were subjected to northern blotting analysis as previously described (Xiao *et al*, 1991). Human 24-hydroxylase cDNA fragments (Chen *et al*, 1993) were labeled with α-[$^{32}$P]dCTP (3000 Ci/mmol, DuPont-NEN, Boston, MA) using a random priming kit purchased from Boehringer Mannheim (Indianapolis, IN).

## RESULTS

**Endogenous and Overexpressed VDR and RXR from Epidermal Keratinocytes Bind *in Vitro* to Vitamin $D_3$-Responsive Element (DR3) as VDR-RXR Heterodimers** To determine how epidermal VDR interacts with VDRE(DR3), we have examined keratinocyte nuclear extracts (8 μg) prepared directly from adult human epidermis by gel mobility shift assays. As shown in **Fig. 1*A***, incubation of P$^{32}$-labeled oligonucleotide probes containing wild-type VDRE with the nuclear extracts resulted in complexes "A" *(lane 1)*. Formation of these complexes was reduced when increasing amounts of unlabeled VDREwt were used as competitors *(lanes 5–7)*. VDREmut, which contains mutations in both half-sites of VDRE, however, did not compete complexes A *(lanes 2–4)*, suggesting that these complexes correspond to proteins specifically recognizing the half-sites in VDRE(DR3).

To further analyze the protein content of the A complexes described above, immunologic gel mobility supershifts were performed using antibodies specific to VDR or RXRs **(Fig 1*B*)**.

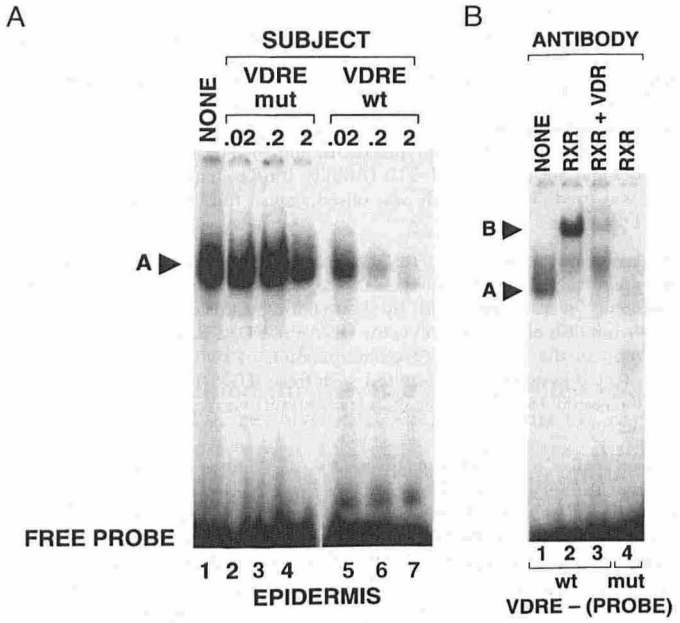


**Figure 1. Endogenous VDR and RXRs bind to VDRE(DR3) as heterodimers but not homodimers.** Gel electrophoretic mobility shift analysis of nuclear extracts from adult human epidermis using $^{32}$P-labeled DR3 as probe; resolved complexes are marked by *labeled triangles on the left side* of gels. *(A)* Competition for formation of specific VDRE-bound complexes. Types and amounts (pmol) of unlabeled competitor DNA (equivalent to 1, 10, 100-fold greater than labeled probes) are indicated at the *top* of the figure. *(B)* Gel mobility supershift assay using RXR- or VDR-specific antibodies. Types of antibodies used are shown at the *top*, and those of labeled probes, at the *bottom* of the gel.

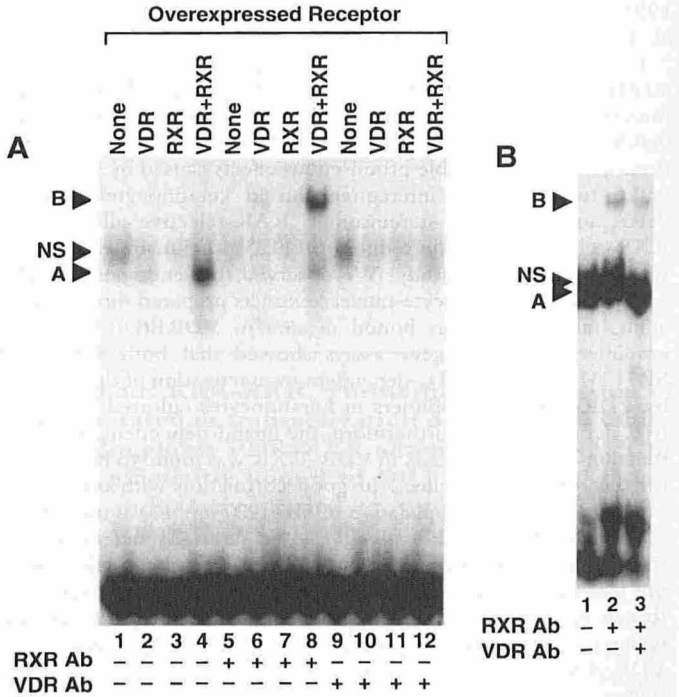


**Figure 2. Endogenous and overexpressed VDR and RXR in cultured keratinocytes bind to VDRE(DR3) as VDR-RXR heterodimers but not homodimers.** Gel mobility supershift analysis of overexpressed *(A)* and endogenous *(B)* VDR and RXR in cultured KCs using VDRE(DR3) as a probe. Types of overexpressed receptors are indicated at the *top* and types of antibodies used, at the *bottom*. Two micrograms *(A)* or 12 μg *(B)* of nuclear extracts were used in each reaction.

Addition of mouse monoclonal RXR antibodies, which recognize an epitope located in the DE region of RXR, into the binding reaction mixtures during post-incubation supershifted the A complexes *(lane 1)* to give complexes "B" *(lane 2)*. This result indicates that the specific A complexes contain RXRs. Pre-incubation of epidermal nuclear extracts with VDR-specific antibodies, which recognize specifically the DNA-binding domain of VDR, strongly inhibited formation of the B complexes *(lane 3)*. Taken together, our data demonstrated that endogenous VDR and RXR bind to the two half-sites of VDRE(DR3) as heterodimers (the A complexes). No complexes with characteristics of RXR-RXR or VDR-VDR homodimers were observed even when 9cRA and/or $D_3$ was added into the binding reactions (data not shown).

In order to know whether at higher concentrations, VDR is able to bind to VDRE(DR3) as homodimers, this receptor was overexpressed alone or together with RXR in cultured KCs from human epidermis. As shown in **Fig. 2*A***, overexpression of VDR alone resulted in complexes A (lane 2) that were supershifted by the RXR antibody to give more B complexes *(lane 6)* and were inhibited by the VDR antibody *(lane 10)*. The increased A complexes apparently resulted from dimerization between overexpressed VDR and limited partner-free endogenous RXRs in cultured KCs. Overexpression of RXR alone, however, did not increases the A complexes *(lane 3)*. Co-overexpression of VDR and RXR produced a synergistic increase in VDR-RXR heterodimers *(lanes 4,8,12)*. Again, no complexes with characteristics of homodimers VDR-VDR or RXR-RXR were observed with overexpressed receptors even in the presence of 9cRA and/or $D_3$ (data not shown). Because only 2 μg of nuclear extracts were used, endogenous VDR-RXR heterodimers present at low levels in cultured KCs were not observed *(lanes 1,5,9)*. When the amount of these extracts used was increased to 12 μg, however, endogenous VDR-RXR heterodimers were readily detected by antibody supershifting, as shown in **Fig. 2*B***. Note that in these extracts, the abundant nonspecific complexes (NS) masked the closely migrating endogenous VDR-RXR heterodimers *(lane 1)*, which were identified by supershifting with the RXR antibody to give complexes "B" *(lane 2)*. These nonspecific complexes were not present in keratinocyte nuclear extracts prepared directly from human epidermis. As expected, the formation of complexes B was significantly decreased by adding the inhibiting VDR antibody *(lane 3)*. In comparison with human epidermis, cultured KCs contain relatively lower levels of VDR-RXR.

**RXR-Specific Ligands, 9cRA and SR11237, Enhance $D_3$-Dependent Transactivation of VDRE(DR3) *via* Endogenous and Overexpressed VDR-RXR Heterodimers in Transfected Epidermal Keratinocytes** To assess whether RXR ligands are able to regulate VDRE(DR3) *via* VDR-RXR heterodimers identified by *in vitro* binding **(Fig 1*B*)**, reporter gene VDRE(DR3)-tk-CAT was introduced by lipofection into normal KCs from adult human epidermis. KCs were cultured in defined serum-free media KGM without significant retinoid and $D_3$ activity. As shown in **Fig. 3*A***, $D_3$ induced CAT activity by about 100%. Although 9cRA alone did not significantly induce CAT activity, 9cRA together with $D_3$ produced a 230% increase in CAT activity. Overexpression of VDR further increased CAT activity over the endogenous receptor activity in response to $D_3$. Cells transfected with the VDR expression vector and the reporter gene were also treated with $D_3$ plus 9cRA. 9cRA caused a further increase of CAT activity over that induced by $D_3$. Synergism between 9cRA and $D_3$ also occurred when VDR and RXR were co-overexpressed, confirming the direct contribution of RXR to the ligand inducibility of the VDR-RXR heterodimers. Comparable results were obtained in similar experiments done with RXR-selective SR11237 (0.1 μM) **(Fig 3*B*)**. As a comparison, no synergism between RAR-selective CD367 and RXR-selective SR11237 was detected in transactivation of βRARE(DR5) by endogenous RAR-RXR heterodimers at a suboptimal activation concentration (0.1 nM) of CD367 **(Fig 3*C*)**. At an optimal activation concentration (1 nM) of

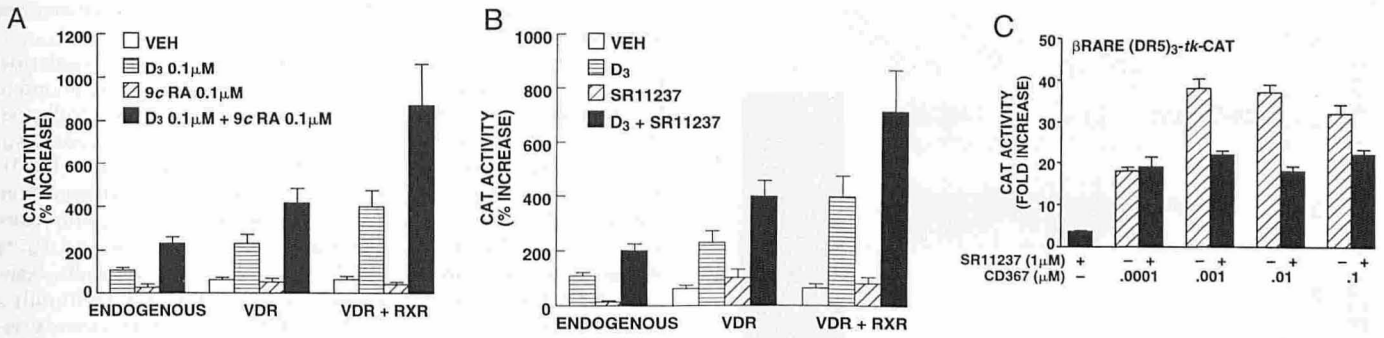


**Figure 3. RXR-specific ligands enhance $D_3$-dependent transactivation of VDRE(DR3) but not RAR ligand-dependent transactivation of βRARE(DR5).** KCs were transfected with 2 μg of VDRE(DR3)-tk-CAT *(A,B)* or βRARE$(DR5)_3$-tk-CAT *(C)* and then treated with vehicle (0.1% ethanol) or indicated ligands for 48 h before measuring CAT activity. The *y* axis represents relative CAT activity expressed as % increase *(A,B)* or fold induction *(C)* over the basal activity of reporter genes in cells treated with vehicle. The *x* axis shows types of expression vectors co-transfected *(A,B)* or types and concentrations of ligands *(C)* used to treat cells. Data are shown as means ± SEM (n = 4).

CD367, SR11237 caused a 40% inhibition of CD367-induced CAT activity. Taken together, our data clearly demonstrate ligand responsiveness of RXR and its direct involvement in $D_3$ signaling over VDRE(DR3), in contrast to the lack of this response in retinoid-mediated signaling over βRARE(DR5).

**AF-2 of RXR Is Involved in VDR-RXR Heterodimer-Mediated Synergistic Induction of VDRE(DR3) by RXR Ligands and $D_3$** To know whether the AF-2 domain of RXR is involved directly in transactivation of DR3, dnRXR was analyzed. dnRXR was generated by deleting a C-terminal 19-amino acid sequence (Xiao *et al*, 1995). This deletion covers a region that has been shown to be important for AF-2, but not ligand-binding and dimerization functions or interaction of RXR with DNA (Durand *et al*, 1992; Nagpal *et al*, 1993; Durand *et al*, 1994). To ascertain whether this deletion affects the ability of RXR to form heterodimers with VDR in binding to VDRE(DR3), wild type RXR or dnRXR was overexpressed in KCs and analyzed by gel mobility shift assays. **Figure 4*A*** shows that dnRXR binds as efficiently as wild-type RXR to VDRE(DR3) in the form of VDR-RXR heterodimers *(lanes 4 and 5)*. This binding was significantly inhibited by the VDR-specific antibodies that recognize the DNA-binding domain of VDR (lanes 9 and 10). These data clearly indicate that deletion of this 19-amino acid AF-2 does not alter the DNA-binding or the dimerization function of RXR. Reporter gene assays shown in **Fig. 4*B***, however, revealed that when dnRXR was overexpressed, either D3 alone or D3 plus RXR-selective SR11237 was not able to activate reporter gene. In VDR and dnRXR co-overexpressing cells, reporter gene activity was increased by $D_3$, but its level was lower than that in VDR and wild-type RXR co-overexpressing cells. Also in these cells, a synergism between $D_3$ and SR11237 is much lower than that in VDR and wild-type RXR co-overexpressing cells. The remaining $D_3$-SR11237 synergism observed in VDR and dnRXR co-overexpressing cells may be caused in part by heterodimers formed between overexpressed VDR and endogenous wild-type RXR that is present in excess relative to endogenous VDR. Taken together, our results suggest that RXR AF-2 is directly involved not only in transactivation of VDRE(DR3) by $D_3$-liganded VDR-RXR heterodimers but also the response of these heterodimers to RXR ligands.

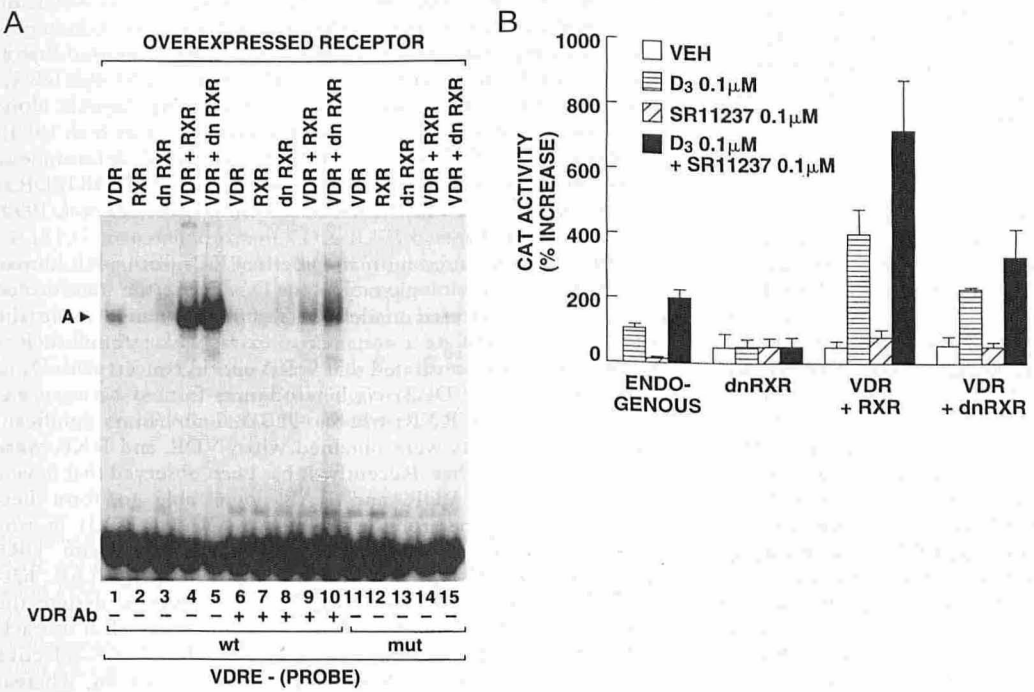


**Figure 4. Deletion of AF-2 impairs the ability of RXR to transactivate but not to bind VDRE(DR3) as VDR-RXR heterodimers.** *(A)* Gel electrophoretic mobility shift analysis of the binding of VDR-dnRXR to VDRE(DR3). Types of keratinocyte nuclear extracts (2 μg) containing overexpressed receptors are shown on the *top*, and those of DNA probes, at the *bottom*. *(B)* CAT reporter gene assays in dnRXR-expressing KCs. The *y* axis represents relative CAT activity expressed as % increase over the basal activity of reporter genes in cells treated with vehicle. Data are shown as means ± SEM (n = 4). The *x* axis shows types of expression vectors co-transfected with the reporter gene (VDRE(DR3)-tk-CAT).

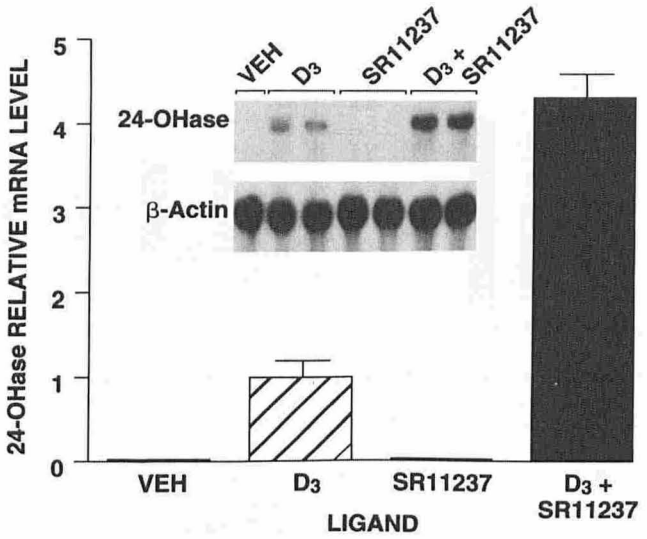


**Figure 5. RXR-selective SR11237 synergistically enhanced $D_3$-induced 24-hydroxylase mRNA levels in mouse epidermis.** Mice were treated by topical application of vehicle (acetone), $D_3$ (32 nmol), SR11237(80 nmol), or $D_3$ (32 nmol) plus SR11237 (80 nmol) to their skin. Twenty micrograms of total RNA from mouse epidermis were analyzed by Northern blotting assays. A representative blot is shown in the panel. The *y* axis represents relative levels of 24-hydroxylase (24-OHase) mRNA by taking the level induced by $D_3$ alone as one unit. Data are shown as means ± SEM (n = 4). The *x* axis shows ligands used to treat mouse skin.

**RXR-Selective SR11237 Synergizes with $D_3$ in Induction of $D_3$-Dependent 24-Hydroxylase Gene Expression in Mouse Epidermis *In Vivo*** In order to ascertain whether the aforementioned ligand synergism *in vitro* reflects the properties of the VDR-RXR heterodimers present at physiologic concentrations in epidermis *in vivo*, we examined effects of SR11237 and $D_3$ on the $D_3$-responsive 24-hydroxylase gene (Chen *et al*, 1993, 1994; Ohyama *et al*, 1994a, 1994b). Normal C57BL/6 mice were treated topically with vehicle (100% acetone) or $D_3$ and/or SR11237. Total RNA was prepared from mouse epidermis and subjected to northern blotting analysis using 24-hydroxylase cDNA as a probe. As shown in **Fig. 5**, 24-hydroxylase mRNA was not detected in the vehicle-treated epidermis. $D_3$ alone induced 24-hydroxylase mRNA levels whereas SR11237 alone had no effect. Interestingly, a large synergistic induction of this gene was observed when both ligands were applied.

## DISCUSSION

In this study, we found that endogenous VDR and RXR in epidermal KCs bound *in vitro* to VDRE(DR3) predominantly as VDR-RXR heterodimers. In transfected KCs, RXR ligands including 9cRA and SR11237 synergized with $D_3$ to transactivate VDRE(DR3) *via* endogenous and overexpressed VDR-RXR heterodimers. Either of these two RXR ligands alone, however, had no effect. In contrast, the same RXR-selective ligand, SR11237, did not synergize with the RAR-selective ligand, CD367, to activate endogenous RAR-RXR heterodimers over βRARE(DR5). The finding that RXR is a silent partner of RAR is similar to data obtained from studying overexpressed RAR-RXR heterodimers using RAR-selective Am580 and TTNPB and RXR-selective LG69 (Kurokawa *et al*, 1994; Forman *et al*, 1995). In addition, we compared the transactivation and DNA-binding functions of the wild-type RXR with those of a RXR mutant lacking AF-2. Results from this comparative study demonstrated that the AF-2 domain of RXR contributed in part to transactivation of VDRE(DR3) by VDR-RXR heterodimers in response to $D_3$ and SR11237 but was dispensable for interaction between the heterodimers and VDRE(DR3).

Our *in vitro* binding study clearly showed that human epidermal KCs express VDR and RXR proteins. Endogenous VDR bound to VDRE(DR3) as VDR-RXR heterodimers but not homodimers. We also found that endogenous VDR and RXR levels were lower in cultured KCs than those in epidermis so that the VDRE(DR3)-bound endogenous VDR-RXR heterodimers in extracts from cultured cells were masked by co-migrating nonspecific complexes. The use of RXR-specific antibodies, however, allowed us to demonstrate endogenous VDR-RXR in these cultured cells. Raising levels of VDR by its overexpression in KCs resulted in only a slight increase of VDR-RXR heterodimers, whereas overexpression of RXR did not do so. Co-overexpression of both receptors, however, produced a synergistic increase of VDR-RXR heterodimers **(Fig 2)**. These observations indicate that endogenous levels of RXR are relatively higher than those of VDR in cultured KCs and that VDR-RXR heterodimers are the major receptor forms binding efficiently to VDRE(DR3) regardless of the levels of each receptor type. In other words, VDR requires RXR for binding to VDRE(DR3) in the keratinocyte cell context.

RXRs are also known to be involved in retinoid signaling through formation of RAR-RXR heterodimers and RXR homodimers. The dominant ligand responsiveness of RXR was found only with RXR homodimers that transactivate through binding to RXRE(DR1) (Zhang *et al*, 1992). In contrast to the ligand responsiveness of RXR homodimers upon RXRE, however, we did not observe the ligand responsiveness of RXR in RAR ligand-dependent transactivation of DR5 by endogenous RAR-RXR heterodimers, most likely due to RAR-induced allosteric conformational changes blocking the access of ligands to RXR in the DR5-bound heterodimers (Kurokawa *et al*, 1994; Forman *et al*, 1995).

Whether the aforementioned allostery exists in VDR-RXR heterodimers bound to VDRE(DR3) has not been clear due to conflicting observations from different cell lines. In the case of VDR and RXR co-overexpressed in transformed cell lines, Carlberg *et al*. (1993) found that in *Drosophila* SL-9 cells, DR3-containing reporter genes were synergistically activated by 9cRA and $D_3$ used together. This synergistic effect was also observed in rat UMR106 cells (Sasaki *et al*, 1995). In contrast, in CV1, COS-7, and ROS 17/2.8 cells, $D_3$ alone was sufficient to induce strong transactivation of VDRE(DR3) by the heterodimers, whereas 9cRA at high concentrations (1 μM) exerted inhibitory effects on this $D_3$-induced transactivation (MacDonald *et al*, 1993). In experiments utilizing endogenous receptors, it has been reported that in MCF-7 cells, $D_3$ and 9cRA synergistically induced a VDRE(DR3)-driven reporter gene whereas either of the two ligands alone produced a minimal induction of the reporter (Carlberg *et al*, 1993). In contrast, in COS-7 and ROS 17/2.8 cells, 9cRA did not alone induce or enhance $D_3$-induced transactivation of VDRE(DR3) through endogenous VDR-RXR heterodimers (Ferrara *et al*, 1994; Schrader *et al*, 1995).

In this study, we used normal epidermal KCs from adult human skin, one of the physiologic targets for $D_3$. Unlike the transformed cell lines, KCs cultured in defined serum-free media retain the potential to differentiate in response to extracellular stimuli such as $D_3$. We clearly demonstrated that 9cRA acts in concert with $D_3$ to transactivate VDRE(DR3) *via* heterodimers formed between endogenous VDR and RXR, whereas 9cRA alone had no significant effect. Similar results were obtained when VDR and RXR were overexpressed together. Recently, it has been observed that *in vitro* translation-derived VDR and RAR were able to form heterodimers. These heterodimers bound to VDRE(DR3) *in vitro* albeit with relatively low affinity in comparison with DR6 (Schrader *et al*, 1994). We did not observe VDR-RAR heterodimers bound to VDRE(DR3) *in vitro*, however, despite the presence of RARs in epidermal and keratinocyte nuclear extracts (Xiao *et al*, 1995; data not shown). Similar to 9cRA, RXR-selective SR11237 plus $D_3$ produced a synergistic transactivation, whereas

RAR-selective CD367 did not enhance $D_3$ activity (data not shown), excluding the possibility that VDR-RAR heterodimers might be responsible in part for the synergism observed *in vivo* with 9cRA, which also is an agonist for RARs (Levin *et al*, 1992) and undergoes isomerization into RAR-selective all-*trans*-RA in cultured KCs (Xiao *et al*, 1995).

The activation function-2 (AF-2) of RXRs has been localized to its C terminus (Durand *et al*, 1992; Nagpal *et al*, 1993; Durand *et al*, 1994, Leng *et al*, 1994, 1995; Xiao *et al*, 1995). Deletion of a 19-amino acid sequence from the RXR$\alpha$ C terminus impaired its transactivation but not DNA-binding, ligand-binding, and dimerization functions within RXR homodimers and RAR-RXR heterodimers ( Durand *et al*, 1992; Nagpal *et al*, 1993; Durand *et al*, 1994; Xiao *et al*, 1995). Here in the $D_3$-signaling pathway, we found that the loss of the 19-amino acid AF-2 did not significantly affect the ability of RXR$\alpha$ to dimerize with VDR and bind to VDRE(DR3) as heterodimers *in vitro*. Expression of such a mutant, dnRXR, however, inhibited transactivation of VDRE(DR3) by the endogenous VDR-RXR heterodimers in response to $D_3$ as well as the $D_3$-SR11237 cooperativity. This inhibition is most likely due to formation of heterodimers between dnRXR and endogenous VDR with the resulting transactivation-defective VDR-dnRXR competing with endogenous wild-type VDR-RXR for binding to target VDRE(DR3). Consonant with this observation, heterodimers formed between dnRXR and VDR co-overexpressed in KCs showed reduced transcriptional activity over VDRE(DR3). Thus, in the absence of RXR ligands, the RXR AF-2 in VDR-RXR cooperates with that of liganded VDR in transactivation. Further binding of RXR ligands to RXR may change the conformation of VDR-RXR and facilitate the interaction between VDR-RXR and general transcription factors such as TIF-1 and TFIIB (Blanco *et al*, 1995; LeDouarin *et al*, 1995), resulting in optimal transactivation. 24-Hydroxylase catalyzes 24-hydroxylation of $D_3$ in $D_3$ catabolism (Ross *et al*, 1994). Its expression is induced at the transcriptional level by $D_3$ in transformed human and rat cell lines (Chen *et al*, 1993; Nishimura *et al*, 1994) as well as rat kidney and intestine (Armbrecht and Boltz, 1991; Shinki *et al*, 1992). This induction has been found to be controlled through interaction between VDR-containing complexes and VDRE(DR3) present in the 24-hydroxylase promoter (Ohyama *et al*, 1994, Hahn *et al*, 1994; Zierold *et al*, 1994, 1995). We also found that the human 24-hydroxylase gene can be induced by $D_3$ in human epidermis *in vivo* and KCs cultured *in vitro* (Kang *et al*, 1997), suggesting that this regulation is conserved *in vivo* among mammals. We used this naturally occurring endogenous gene as a reporter to analyze roles of RXR and its ligands in regulating the $D_3$-signaling pathway in epidermis *in vivo*. When topically applied to mouse epidermis, $D_3$ and RXR-selective SR11237 synergistically induced this gene. This induction was most likely mediated by VDR-RXR heterodimers but not RXR homodimers because SR11237 alone did not do so and because activity of the homodimers was not detected in epidermis (J-H. Xiao *et al*, manuscript in preparation). Most interestingly, $D_3$ and SR11237 cross-talk through VDR-RXR heterodimers in epidermis whereas regulation of RAR-ligand-responsive gene CRABPII by RAR-RXR heterodimers was not augmented by RXR ligands (data not shown). This phenomenon raises the possibility that RXR-selective agonists in combination with low doses of $D_3$ may be beneficial in treating skin diseases by eliminating potential systemic side effects caused by higher doses of $D_3$, but without activating the RAR-RXR heterodimer-mediated signaling pathway.

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